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Synthesis, bioanalysis and biodistribution of photosensitizer conjugates for photodynamic therapy

Tyler GSt Denis^{1,2} and Michael R Hamblin^{2,3,4,*}

¹Department of Chemistry, Columbia University, NY, USA

²The Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, USA

³Department of Dermatology, Harvard Medical School, Boston, MA, USA

⁴Harvard-MIT Department of Health Sciences & Technology, Cambridge, MA, USA

Abstract

Photodynamic therapy (PDT) was discovered in 1900 by Raab, and has since emerged as a promising tool for treating diseases characterized by unwanted cells or hyperproliferating tissue (e.g., cancer or infectious disease). PDT consists of the light excitation of a photosensitizer (PS) in the presence of O₂ to yield highly reactive oxygen species. In recent years, PDT has been improved by the synthesis of targeted bioconjugates between monoclonal antibodies and PS, and by investigating PS biodistribution and PD. Here, we provide a comprehensive review of major developments in PS-immunoconjugate-based PDT and the bioanalysis of these agents, with a specific emphasis on anticancer and antimicrobial PDT.

Photodynamic therapy

Photodynamic therapy (PDT) is without doubt best known for its anticancer applications [1]. However, unlike most conventional anticancer modalities, PDT had a serendipitous beginning in the destruction of microbes. In 1900, Raab observed the accidental eradication of *Paramecia* spp. protozoa when these cells were stained with acridine orange and exposed to intense white light [2]. Immediately thereafter, this phenomenon was observed to require the presence of oxygen (termed photodynamic action) and was investigated for potential biomedical applications. Initial experimentation demonstrated that PDT had promise in the destruction of cancerous tissue [3–5], but the anticancerous effects would only be realized by the mid-20th century, as the two world wars redirected PDT researchers to the war efforts. However, in the 1960s interest in PDT was rekindled and by the end of the 20th century, PDT was US FDA-approved for the treatment of bladder, dermatological and gastrointestinal malignancies, noncancerous dermatological diseases and age-related macular degeneration of the retina (characterized by excessive growth of vascular tissue). At present, PDT is attracting attention as a prospective alternative to antimicrobials, especially when the pathogens are resistant to conventional antibiotics [6].

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*Author for correspondence: hamblin@helix.mgh.harvard.edu.

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Diseases that are good candidates for PDT are typically characterized by hyperproliferation [7]. This should not come as much of a surprise, as PDT is routinely used in dermatology for benign disease (e.g., acne and psoriasis); skin is characterized by high cell turnover rates relative to other tissue types. Likewise, cancer is characterized by hyperplasia, age-related macular degeneration is characterized by excessive endothelial growth and microbes grow at faster rates than the mammalian cells. Hyperproliferating tissues preferentially accumulate the light-sensitive dyes used for PDT known as **photosensitizers** (PS). This is because hyperproliferating cells require a greater amount of nutrients relative to their slow-growing counterparts and tend to cause neovascularization to fulfill this requirement. The PS are preferentially accumulated due to abnormal blood supply, lack of lymphatic drainage and several other factors that may also be involved [8]. Like all chemotherapeutic drugs, PS may also accumulate in healthy tissue, but because the photodynamic effect only occurs where light of the appropriate wavelength and intensity is delivered to specific tissues, the general toxicity to the host is likely to be drastically reduced. One of the key features of PDT is therefore the dual selectivity afforded by PS localization in lesions, in combination with anatomically confined illumination [9].

While the spatial selectivity afforded by light excitation of PS is a major positive feature of PDT, light-dependent cytotoxicity is a double-edged sword. By far the greatest drawback of PDT, impeding its clinical development, is skin photo-toxicity when exposed to sunlight or even indoor lighting [10]. This side effect has been of particular concern when PS are intravenously administered to patients and animals as part of a cancer treatment. Epithelial cells of the skin (epidermis) also accumulate porphyrins and other cyclic tetrapyrrole PS, such that ambient light exposure from the sun or artificial light may elicit undesired photodynamic cytotoxicity, leading to skin burns. Moreover, visible light in the red portion of the electromagnetic spectrum (630–650 nm) only penetrates tissue to approximately 1–2 cm. Consequently, recent chemical efforts have focused on the synthesis of PS with absorption in the infrared (IR) or, more recently, near-IR (NIR) portion of the electromagnetic spectrum. Despite this, such endeavors have not always been as easy, since NIR (more specifically, IR) light is less capable of inducing photonic transitions and, rather, induces molecular vibrations.

In an effort to increase host PS accumulation specificity, and reduce unwanted PDT side effects, significant effort has been devoted in recent years towards the synthesis and characterization of bio-conjugates and, specifically, immunoconjugates with PS [11]. To date, there is only a single case of PS antibody conjugates in use in the clinic. Success in *in vitro* and *in vivo* experimentation suggests, however, that, just as PDT is gaining ground in the medical community, so will **photoimmunoconjugates** (PICs) as an extension of PDT and as a means of combating malignancies, cancers, infections and other ailments. Despite this, there is one considerable drawback preventing these conjugates, and all other monoclonal antibody (mAb) conjugates, from gaining clinical ground in the fight against cancer: mAbs are large relative to small-molecule drugs and this large size limits accumulation of PICs into dense, deep-seated and frequently poorly vascularized malignancies. Here, we present a review on major developments in PS immunoconjugates and their application in PDT, with an emphasis on their bioanalysis.

Photosensitizers

Photophysics

PDT involves the light excitation of a PS. The PS exists in a ground singlet state, such that upon excitation by a photon of suitable energy, a single electron in the highest occupied molecular orbit is promoted to the lowest unoccupied molecular orbit, preserving the spin orientation of the original species and bringing the PS to an excited singlet state [12]. At this

point, several distinct physical processes may occur; however, the most important for PDT is a reversal of the spin orientation of the promoted electron, bringing the PS to an excited triplet state. Because this electron now has a spin parallel to its partner, the electron may not simply relax, as this would be a violation of the Pauli exclusion principle. Consequently, the triplet state has a significantly longer lifetime than the excited singlet state that allows it to undergo photochemistry. This process is illustrated in a modified Jablonski diagram (Figure 1).

Photochemistry

The PS triplet state is so long-lived that it may react with other triplet molecules in a spin permitted process. Dioxygen (O_2) unusually has a ground triplet state – as evident by its only moderate oxidizing abilities and paramagnetic properties – and triplet O_2 may interact with the triplet-state PS in two distinctly different processes. The type I reaction involves the electron transfer from the triplet-state PS to O_2 , forming the superoxide anion ($O_2^{\cdot-}$), which may then form hydrogen peroxide (H_2O_2), and then hydroxyl radical ($\cdot OH$) and hydroxide ion in the presence of a reducing agent, such as ferrous iron. The type II reaction involves the energy transfer of the triplet state PS to O_2 , forming excited state singlet oxygen (1O_2) [12].

The **reactive oxygen species** (ROS) formed during PDT are responsible for initiating oxidative stress, which may subsequently lead to cytotoxicity and cellular death. The oxidative stress of PDT may also result in the formation of reactive nitrogen species when reacting with nitric oxide, but the chemistry of reactive nitrogen species-mediated PDT is beyond the scope of the present review [13]. It is important to note that several of the ROS generated during PDT are linked to cellular signaling (e.g., H_2O_2) and that the efficacy of PDT may, in part, be linked to a stimulated immune response. The effect of PS localization and its effect on cellular signaling pathways are elaborated on later in this article.

Ideal qualities of a PS

Even though PDT consists of three critically important agents: light, O_2 and PS, the PS is unequivocally the most important of the three. This should not come as much of a surprise as the PS structure dictates the excitation wavelength (desirably in the red and NIR portion of the electromagnetic spectrum, as this corresponds to greater tissue penetration of light) and intensity of light required. The PS structure also affects the solubility, ROS yield and cellular localization and internalization [1].

PS are traditionally large planar aromatic organic compounds derived from a natural product (as is the case with porphyrins, chlorins or bacteriochlorins) or coal-tar-derived chromophores (e.g., phenothiazine dyes). Several inorganic PS, such as titanium dioxide, have been explored for PDT applications as a consequence of their well-known photocatalytic applications. The best PS tend to be deeply colored, reflecting the low energy difference between the highest occupied molecular orbit and lowest unoccupied molecular orbit and, thus, the high propensity for excitation by appropriate photons.

PS are typically designed to meet several demanding chemical and physical properties. On top of these challenges, PS must also exhibit desired biological properties, thus making goal-oriented PS synthesis particularly challenging. The ideal PS should meet the following requirements:

- Excitation should result in production of $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$ (type I reaction) or 1O_2 (type II reaction), or some combination thereof, in appreciable yields;

- Photonic absorption should occur in the deep red and NIR portion of the electromagnetic spectrum. Wavelengths shorter than 600 nm are absorbed and scattered by naturally occurring tissue chromophores (e.g., melanin and hemoglobin). Red light (600–800 nm) corresponding to deep tissue penetration is desirable for biomedical application and is known as the optical window; however, a bathochromic shift in PS absorption should not come at the expense of decreased electronic excitation. Wavelengths longer than 1000 nm stimulate molecular vibrations and are typically absorbed by water, resulting in a localized photothermal effect, while wavelengths between 800 and 1000 nm do not have sufficient energy to excite oxygen to its singlet state;
- Without the application of light, the PS should be photochemically inert (no dark toxicity). No ROS should be generated in the dark. Both intravenous and topical administration of PS in the dark should not adversely affect patients. For anticancer applications, intravenous administration should result in high PS retention in malignant tissue and little to no retention of PS in normal tissue;
- For intravenous administration, the PS chromophore should be hydrophilic with a hydrophobic moiety to facilitate crossing cell membranes. The hydrophobic moiety should not behave as an auxochrome that results in a hypsochromic shift in the PS absorption profile. These PS should be solvated in amphiphilic substances so as to facilitate PS crossing cell membranes;
- While amphiphilic PS with an anionic charge are ideal for anticancer PDT, they are not desirable for antimicrobial PDT. Antimicrobial PS should have a cationic charge, affording efficient binding to the inherent anionic charge of Gram-negative, Gram-positive and fungal cells. Host cells should not retain antimicrobial PS;
- PS should be stable at room temperature for extended periods of time;
- PS should be chemically pure and the synthesis should be straightforward and high-yielding. Synthesis should result in the enantiomeric excess of the desired compound when a chiral center is present.

To date, no PS has satisfied all of the requirements for an ideal PS. With phototoxicity issues occurring in the early years of PDT treatments, it became very apparent that the lack of selective PS accumulation could be hindering widespread PDT application. Selective PS accumulation has been improved by modifying PS chemical structure, but conjugating PS to molecules that selectively bind to target cells (e.g., mAbs) has acquired a good deal of interest from the bioconjugate community.

Preparation & purification of PICs

Conjugation of PS to biomolecules, and specifically to mAbs, relies on the unique or increased presence of a membrane-bound target molecule or receptor on cancerous cells. Because PDT has been predominantly concerned with porphyrin and phthalocyanine-based PS and the chemical modification of these aromatic cyclic systems is well known, the majority of PIC synthesis has been based on cyclic tetrapyrrole PS [14–16]. PIC synthesis can be deceptively facile, as there are significant difficulties in separating and purifying PICs from their unmodified mAb counterparts and from unbound PS. Furthermore, due to the complexity of the mAb tertiary structure, the antigen-binding affinity and the molecular recognition sites may be adversely affected by conjugation and the optical properties of PS may be disrupted during the synthesis. Methods of conjugation are outlined in Figure 2.

PIC preparation

Bioconjugate synthesis is also challenging as antibody modification and purification should not alter antibody function (antigen recognition). Consequently, PICs are characterized by conjugation efficiency (PS/mAb), biodistribution and target specificity of the PIC, and photophysical properties (i.e., absorption spectra) of the modified PS. One problem frequently encountered in the synthesis of PICs is the reproducibility of PIC formation. That is, while PICs are reproducibly synthesized, both the yields and the PIC products (in terms of mAb/PS ratios) can sometimes vary considerably. Nevertheless, two chief techniques for direct PIC preparation have emerged: the use of a *N*-hydroxysuccinimide (NHS) PS ester (which is discussed frequently in this text) and the use of a pyridyldithiopropionic acid NHS ester or a maleimide-activating group [14]. The chemical principle behind PS–mAb conjugation is trivial and requires only a basic understanding of chemical mechanisms. Several amino acid residues (e.g., lysine, cysteine, threonine, serine, glutamine and arginine) of mAbs possess the chemical ability to serve as nucleophiles, and these side chains only become suitably nucleophilic in optimized chemical conditions. Most PS possess carboxylic acid residues (see hematoporphyrin) and these may serve as suitable electrophiles once a suitable leaving group is formed (via esterification). By formation of the appropriate aforementioned ester on the PS, an amino acid side chain of the mAb attacks the electrophilic carbon of a carbonyl, forming a tetrahedral carbonyl intermediate that later expels the corresponding NHS, pyridyldithiopropionic acid NHS ester or maleimide leaving groups, allowing for facile ester, amide or thioester bond formation.

The presence of NHS esters leads to conjugation at the site of lysine residues. Lysine is relatively abundant in mAbs, compared with other amino acid residues, and there may be as many as 100 lysine residues in a mAb. Random conjugation between PICs and PS using the NHS method thus leads to greater conjugation efficiency (i.e., moles of PS/moles of mAb in the product), statistically explained by the presence of excess lysine residues. But as the degree of conjugation increases (i.e., more PS conjugate to a single mAb), PIC antigen-binding affinity decreases, due to the presence of large PS chromophores in higher abundance producing a heterogeneous environment for protein folding. The mAb portion of the PIC may thus fold in a drastically different manner compared with the original mAb. The only potential downside of the pyridyldithiopropionic acid NHS ester or conjugation of a maleimide leaving group to an mAb is that both rely on thiol groups of cysteine residues in the mAb to serve as nucleophiles. The same cysteines are usually oxidized as cystines, forming disulfide bridges between mAb domain subunits, and, thus, play imperative roles in the stabilization of mAb chains and ensure appropriate conformation of mAbs. Because thiols are less abundant, it is less likely that large changes in the heterogeneity of the mAb will occur. Nevertheless, the issue of mAb destruction due to consumption of necessary cysteine thiol groups has been circumvented by thiolation of mAbs prior to conjugation.

In 1975, Köhler and Milstein were able to make large quantities of purified mAbs from hybridomas, for which they received the 1984 Nobel Prize in Medicine or Physiology [17]. The formation of mAbs that can selectively bind to cancerous cells inspired early PDT investigators to formulate methods of attaching PS to immunoglobulin G mAb substrates. Due to the known chemistry of carboxylic acid compounds and the stability of amides in the cellular environment, the first PIC synthesis involved direct linkage of PS with a carboxylic acid moiety to amino groups on the mAb. In 1983 Mew *et al.* published one of the first studies involving the direct linkage of hematoporphyrin (Figure 2) to mAbs against the DBA/2J myosaroma M-1 antigen [18]. Hematoporphyrin was chosen as an ideal PS substrate for conjugation, because the presence of carboxylic acids allowed the formation of amide bonds with amino-groups on the mAb. Use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride converted the hematoporphyrin carboxylic acids to activated carboxyls prone to nucleophilic attack. While Mew and

colleagues did not report which amino acid residues behaved as nucleophiles, one can imagine that free amino group-bearing amino acids such as lysine could form amide adducts with the activated hematoporphyrin. More importantly, using an ELISA assay, Mew *et al.* demonstrated that binding affinity of the mAb substrate was not affected and that the hematoporphyrin-conjugated mAb was capable of hemolyzing erythrocytes just as successfully as free hematoporphyrin. In Mew *et al.*'s column preparation of the hematoporphyrin PIC, it was determined that less than 10% of the hematoporphyrin present interacted with the mAb in a noncovalent interaction (i.e., excess hematoporphyrin remained in the PIC preparation). Mew and colleagues' PIC led to suppression of M-1 myosarcoma (antigen-positive) cell growth and had no effect on the antigen-negative C57BI/6J lymphoma EL4 cell line, corroborating that the killing effect was dependent on the antibody-binding specificity.

Similar PIC synthesis by direct conjugation involving the presence of a carboxyl moiety has been used by our group to link the chimeric antihuman EGF receptor mAb, known as C225, to the fluorescent cyanine dye Cy5.5 and to the PS chlorin e6 [19]. The PIC adduct allowed for photodiagnosis as well as PDT of premalignant cells in a hamster cheek pouch carcinogenesis model. The PIC efficiently destroyed target premalignant cells without affecting the viability of normal cheek epithelium. Direct conjugation has also been used to conjugate *meso*-tetraphenylporphyrins to non-mAb-targeting agents. These are often small molecules that are recognized by specific cell-surface receptors such as folic acid and oestradiol. Recently, a group led by Mitsunaga conjugated a silicon-phthalocyanine derivative, the NHS ester IRDye 700DX, to the commercially available mAbs trastuzumab and panitumumab, to target human EGFR 2 and 1, respectively, for photo-thermal destruction of cells [20]. In a slightly basic solution, amino acid residue nucleophiles attacked the NHS ester, eliminating the NHS group. Unfortunately, only three PS conjugated to a single mAb and mAb binding affinity was slightly reduced. In spite of this, Mitsunaga's PICs destroyed breast cancer cell lines known to overexpress EGFR. This was used in an *ex vivo* murine tumor model that effectively reduced cancer cell viability. Interestingly enough, the PICs were exclusively toxic when bound to cells and irradiated by light; there was no dark toxicity and when PICs were added to a tumor cell line that did not express either growth factor, no cell death was noted even with irradiation.

The second and more popular means of synthesizing PICs involves the attachment of PS to a polymeric carrier that is then attached to a mAb. This affords a high PS:mAb ratio without significantly altering mAb structure. Oseroff *et al.* pioneered this technique in 1996 by first coupling chlorin e6 to dextran polymers and then conjugating the PS-loaded dextran to the anti-T-cell mAb, anti-Leu 1 [21]. A mAb/PS molar ratio of 1/36 was reported and the photophysical properties (absorption spectra and quantum yield) did not change relative to chlorin e6 alone. These findings have been also reported by Mayo *et al.* in the conjugation of verteporfin to VEGF [22]. This indicates that the loading was such that the local environment in which the photochemical processes of PDT occur was significantly altered. As proof that the PIC method is superior to conventional PDT specificity (i.e., therapy without use of a mAb) free chlorin e6 was 100-times less effective at killing cells than the conjugate system. This impressive study then calculated that approximately 10^{10} molecules of $^1\text{O}_2$ were needed to kill each single leukemia cell in a model using the human T-cell leukemia line HPB-ALL [21].

Fab' fragments were conjugated with mesochlorin e6 monoethylene diamine PS using the indirect approach via a *N*-(2-hydroxypropyl) methacrylamide copolymer-drug linker [23]. This system was evaluated against ovarian cancer. Comparing PS alone, PS with copolymer and PS with copolymer and the Fab' fragment, it was determined that the PS alone and PS combined with copolymer were less effective than the PS, copolymer and Fab' fragment.

This was explained by the enhanced biorecognition of PS when conjugated to Fab' fragments observed, as determined by confocal microscopic analysis, which noted that PICs localized and internalized in OVCAR-3 cells.

A more recent example of the indirect linking approach was used for the synthesis of a modular system for PIC-mediated PDT. Using a 5:1 fullerene hexakis adduct with five malonate spacers, Ranchan *et al.* were able to link two separate PS molecules (pyropheophorbide-a) to each of the malonate spacers, such that each fullerene adduct possessed a total of ten PS [24]. An additional, longer malonate spacer was used to attach the modified fullerene to the mAb rituximab (anti-CD20 receptor protein). Linkage of pyropheophorbide-a to the antibody only slightly lowered the absorption intensity of the fullerene adduct and led to no batho- or hypsochromic shift in the PS absorption profile. In spite of this decreased intensity, confocal microscopy studies determined that the PIC specifically recognized CD20-positive B-lymphocytes and ignored Jurkat cells. Other examples of indirect linking of PS to mAbs are discussed below.

PIC purification

The objective of PIC purification is twofold. First, it is imperative to separate PICs from their constituent, unreacted mAbs, PS and linkers, in addition to chemical impurities (solvents and reagents that alter PIC function and/or are inherently toxic) used in the coupling of mAbs and PS. One would presume that purification of PICs from PS is relatively easy as they differ greatly in terms of molecular size. While purification from excess PS is not always simple, generally speaking it is easier than the separation of PICs from their corresponding mAbs. Second, the desired PIC (i.e., a PIC with a desired PS/mAb ratio) must be separated from PICs of undesired PS/mAb ratios and, in the case of PEGylated PICs, mAbs and PICs with high degrees of PEGylation.

Column chromatography has been routinely used to purify PICs and is advantageous in that it is capable of easily separating PICs from impurities and PS (which tend to elute first) [16,25]. Hamblin *et al.* used column chromatography to purify hematoporphyrin cationic immuno-conjugates raised against NIH:OVCAR-5 ovarian cancer cells [25]. 0.1% SDS was needed to prevent aggregation of PICS in eluents. Fast-eluting fractions were high-molecular weight and possessed chemical entities that absorbed light at 280 and 380 nm (corresponding to aromatic amino acids of proteins and PS, respectively). The next batch of fractions (corresponding to slower eluting peaks) were lower megawatt compared with fast-eluting fractions and possessed absorption at 380 nm with little absorption at 280 nm. This absorption profile suggests that fractions were primarily composed of mAbs and PICs of low PS/mAb ratios. Using a column chromatogram coupled with a UV/Vis spectrophotometer, it is possible to estimate the PS/mAb ratios. Hamblin and colleagues determined that using their conjugation procedure, six PS molecules conjugated to substrate mAbs [25]. That being said, only gel electrophoresis can be used to definitively demonstrate that covalent bond formation between mAbs, linkers, PS and other additions is occurring; UV/Vis spectrophotometry could lead to incorrect estimations of PIC size due to aggregation of unreacted starting material.

Alternatively, gel filtration may be used to separate PICs from unreacted starting materials. Jiang *et al.* used gel filtration to separate reproducibly formed PICs consisting of benzoporphyrin derivative monoacid ring A and mAbs [26]. Jiang *et al.* initially encountered problems as their mAb of choice, mAb derivatives (including a polyvinyl alcohol addition) and PICs had a high affinity to stick to essentially all crosslinked dextrans. However, addition of 0.5% final concentration low-molecular weight polyvinyl alcohol prevented binding of adducts of interests to dextran materials. A column with a bed volume of 68.4 ml was first equilibrated with acetate buffer (0.01 M; pH 5.5). Hemming *et al.* conjugated

photofrin II and benzoporphyrin to anti-carcinoembryonic antigen (CEA) mAbs and used Jiang's method for purification [27].

In the case of PEGylated PICs, purification is needed to remove and discard excessively PEGylated material that may still possess the desired PS/mAb ratios. The Hasan group, which has championed PEGylation for improved PIC formulation, has used column chromatography to successfully purify PEGylated PICs [28]. Column chromatography with 1/1 DMSO/deionized water successfully eluted desired PEGylated PICs and these adducts were so stable that they could be stored in 50% DMSO solutions for up to 4 months at 4°C. For further purification prior to use, PICs were passed through sterile filters of 0.2- μ m pore diameter. A small addition of albumin prevented adsorption of PICs onto filter membranes.

PIC characterization & assessment of function

The important factors to be considered in this section are:

- To what extent is the mAb functionality preserved after conjugation?
- To what extent is the PS functionality preserved after conjugation?
- How stable is the linkage between the mAb and the PS?

Many methods have been developed for characterization and assessment of function of mAbs labeled with radioisotopes to produce radioimmunoconjugates for diagnosis and targeted therapy of cancer. These analytical methods can also be used for **PIC bioanalysis**. It was realized that the conjugation procedure used to prepare radioimmunoconjugates may destroy the immunoreactivity of the antibody; that is, its ability to bind to the relevant antigen. There are at least two aspects to this problem: how much of the antibody is still able to bind to its antigen, and what is the affinity of the remaining immunoreactive antibody? The immunoreactive fraction can be measured using a binding assay under conditions of antigen excess, so that all the mAb that is able to bind to antigen should be bound. The immunoreactive fraction would then be expressed as the amount of bound antibody relative to the total amount applied [29]. A more accurate determination would be obtained if a series of increasing concentrations of antigen were used in the assay. One would then expect the relative amount of bound antibody to approach a plateau value, which would thus define the immunoreactive fraction [30]. In each instance the amount of nonspecific binding must be determined and subtracted from total binding, since nonspecific binding may increase as immunoreactivity decreases. In fact this type of assay has become known as the 'Lindmo assay' after its chief proponent [31].

Affinity is determined by measuring free antigen and antibody-bound antigen at equilibrium over a range of antigen concentrations. The use of several antigen concentrations is essential in order to achieve saturation of antibody binding sites and to overcome heterogeneity of affinity within an immunoconjugate. An important requirement for the techniques of separating antibody-bound and free antigen is that the equilibrium is not disturbed. This has been achieved by methods employing a dialysis membrane, selective precipitation by salt, a second antibody or other precipitant, and by utilizing changes in the fluorescence properties of the antibody or antigen occurring as a result of binding. In general, it has become accepted that the reference method for affinity determination is equilibrium dialysis [32].

It is also possible (and may even be considered likely) that the photophysical and photochemical properties of the PS can be adversely affected by the conjugation procedure. The microenvironment of the PS will undoubtedly change upon conjugation to a large protein, such as immunoglobulin G, and this may result in increased PS aggregation and lower yield of excited singlet and triplet states. However, if the PS was pronouncedly hydrophobic before the conjugation, attachment to a large protein might improve the

microenvironment of the PS. Furthermore, the restricted rotation, consequent upon covalent conjugation, may also reduce excited state yields and lifetimes. The absorbance, fluorescence emission and yield of different ROS after photoexcitation can all be measured and compared with unconjugated PS.

Finally, the stability of the PIC should be investigated, as for the construct to be functionally useful it should exhibit sufficient stability (e.g., in serum) to enable it to accumulate in its target tissue and bind to its cognate antigen. Examples are known of drug–antibody conjugates that have severely reduced serum stability [33]. On the other hand, there is a report of increased serum stability [34].

Bioanalysis of PICs

Even though the biodistribution of PICs has been studied at great length, the bioanalysis of PICs has been explored to a lesser degree. Drugs should typically have a quick clearance from the vascular system yet incubate in an organism for a sufficiently long time to allow binding and localization at an appropriate site. Unfortunately, it has been established that modification of mAbs has two deleterious effects: reducing antibody–target recognition and extending the mAb half-life in the body [35,36]. With an extended half-life of a PIC relative to free mAbs, there is an increased likelihood that PICs will be degraded and free PS will be released into an organism. This would lead to phototoxicity, thus nullifying the PIC synergism.

Several times in this review, the effect of PIC charge on PIC half-life has been noted. Both Hudson and Hamblin independently demonstrated that a cationic charge on PICs reduces photoimmunotherapy. Hudson determined that addition of a cationic charge reduced the efficiency of their PIC system (i.e., neutral PICs are better) [37], while Hamblin and Hasan demonstrated that anionic PICs performed better than cationic PICs *in vivo* [38]. This is rather interesting as previous investigations by Duska *et al.* have demonstrated that intraperitoneal injection of cationic PICs was more efficient than the use of anionic analogues [39]. Interestingly, anionic PICs achieved greater PS loading, superior PS delivery to target tissue and greater half-lives *in vivo*. Cationic PICs accumulated in the lungs, yet the mechanism of this accumulation has yet to be unambiguously elucidated. These initially contradictory findings can be reconciled when it is realized that cationic PICs were superior when administered into the peritoneal cavity or other locoregional delivery method, while anionic PICs were superior when injected intravenously.

While the PICs synthesized are varied, the techniques used to characterize these compounds are few and far between. First and foremost, intrinsic optical properties of proteins and PS have been exploited to determine the number and distribution of conjugates of given PS/ mAb ratios. For example, a report by Linares *et al.* on the synthesis of IL-2-binding mAbs detailed the use of UV/ Vis spectrophotometry to determine protein and PS concentrations at 280 nm and 500–600 nm, respectively, in samples post-synthesis [40]. This technique is advantageous in that the aromatic chromophores of proteins (tyrosine and tryptophan) have distinctly different absorption profiles compared with the significantly bathochromically shifted PS.

In addition to spectrophotometric techniques, significantly more sensitive analytical biological techniques have been employed. Agarose and polyacrylamide gel electrophoresis are advantageous in that, with use of a Coomassie blue staining procedure, proteins may be quantified based on all peptide bonds, rather than the presence of distinct aromatic moieties. The PS are also generally highly fluorescent and can be localized on gels whether bound or unbound to mAbs. Both gel techniques have been employed in elucidating PS/ mAb ratios in varied PIC syntheses. A fine example of the utility of gel electrophoresis in determining PS/

mAb ratios is that of Savellano and Hasan [41]. Nonreducing SDS-PAGE was used, so as to prevent reduction of the disulfide bonds that bind the outer light chains of an antibody as well as the two central heavy chains. Reduction of disulfide bonds splits the chains by destroying the antigen-binding sites and, thus, the functional properties of antibodies. Nonreducing SDS-PAGE allowed Savellano and Hasan to estimate the molecular weight of conjugates. With the aid of densitometric analyses, the relative ratios of conjugates yielded substitution ratios. The Hasan group launched the idea of PEGylation to improve functionality of PICs. They used SDS-PAGE to determine the degree of PEGylation of PICs. Interestingly, on a nonreducing SDS-PAGE, there are a few faint bands at the top of the gel, indicative of highly PEGylated PICs. That being said, three PEGylated species with smaller molecular weights were identified with PEG moiety/PIC ratios of 2, 1 and 0.

Distantly related to PICs, Zdobnova *et al.* produced inorganic semiconductor nanocrystal (quantum dots [QDs]) for microscopic visualization of cancerous cells [42]. QDs consisting of carboxyl-coated cadmium selenide with sizes corresponding to chief absorption peaks of 565 or 605 nm were linked to barstar, the substrate of the enzyme barnase ('Bacterial Ribonuclease'), and linked barnase to anti-EGFR or anti-HER2 scFv mAbs. The idea behind this system is that the barnase-mAb conjugates first attach to cells, and the barstar-conjugated QDs then bind to the already bound receptor system, therefore changing the localized environment of the QDs (and, thus, their respective fluorescence spectra). The group evaluated the efficiency of conjugation of barstar with PS by agarose gel-electrophoresis. Unlike SDS-PAGE, which can determine the spectra of conjugates produced, agarose gel electrophoresis cannot. This is because SDS-PAGE relies on the use of the anionic detergent, SDS, to apply a net anionic charge on proteins. Agarose-gel electrophoresis does not use the same sample preparation, such that the only information on efficiency of conjugation that could be obtained pertained to the degree of which the intrinsically anionic cadmium selenide QD movement along the agarose gel was retarded. As agarose-gel cannot provide the same information on the relative amounts of various ratios of PICs, nor can it specify the ratio of PS/mAb, it is seldom used in the bioanalysis of PICs.

In addition to its purification prowess, HPLC has proven invaluable in both the isolation and purification of PICs. Vrouenraets *et al.* created hydrophilic PS-mAb conjugates, evaluating PIC integrity via SDS-PAGE and UV/Vis-coupled HPLC [43]. Antibodies were radiolabeled with ¹²⁵I, enabling simultaneous detection of HPLC eluant fractions of the PS at 424 nm, and ¹²⁵I-mAbs, with a single channel radioactivity detector. HPLC not only determined that radiolabel recovery was high at >95%, but that passing PIC mixtures through the silica column efficiently separated excess PS from PICs. This is important to note since the presence of excess PS may initiate aggregation of PICs. The same group used HPLC to analyze the integrity of PIC samples under various conditions, akin to the use of SDS-PAGE by Hasan and Savellano [41]. Incubating PICs with human serum for 24 h and passing samples through HPLC produced PIC samples identical in purity and function to controls. This time, spectrophotometric analyses at 280 and 424 nm for peptide bonds and porphyrins, respectively, as mentioned earlier, were used to determine the purity and stability of PIC samples post-chromatographic separation. This underscores the stability of PICs in human serum.

Other HPLC techniques have also been used to purify PICs. Reversed-phase HPLC has been used to determine the hydrophobic character of various PEGylated porphyrin-peptide conjugates by looking at the retention time of individual conjugates [44]. The same group also used reversed-phase HPLC to trace the presence of conjugates in cellular extracts after 24 h. After 24 h, both the presence of the original PEGylated porphyrin-peptide conjugate and the degradation products was detected. All degradation products contained the

porphyrin macrocycle. It was concluded that porphyrin–peptide conjugates are subject to metabolic degradation of HEp2 cells.

MALDI-TOF spectroscopy has shown that PICs or PS–peptide conjugates localize inside of cells. MALDI-TOF analysis of cellular extracts of HEp2 cells demonstrated that after 1 h, PS–peptide conjugates entered cells and remained there for more than 24 h [44]. MALDI-TOF has also been used less elegantly merely to characterize the PICs molecular weight, as MALDI-TOF tends to not fragment large molecules compared with other MS ionization technologies [45].

Photosensitizer localization & biodistribution in PICs

PICs against EGF receptors

In the case of anticancer PDT, malignant cells present different types as well as greater amounts of many surface antigens. On occasion these antigens may be specifically expressed only on tumors or, more commonly, be overexpressed on tumors compared with non-malignant tissues, when they are termed **tumor-associated antigens**. Antibodies against these antigens are easily generated, such that the bulk of PIC-mediated PDT examples have been targeted against malignancy antigens. It should thus come as no surprise that the main target in PIC-mediated PDT is the cell membrane. Attachment of PS at/near the cell membrane via the PIC technique is particularly advantageous in that it leads to oxidation of lipids of the cell membrane, which in turn leads to cellular leakage, a sure and rapid means of inducing cell death.

The Hasan group at the Wellman Center for Photomedicine has, over the past 10 years, focused on targeting the EGFR (Figure 3) [19,28,41,48,52]. EGFR is an attractive target for PDT in that not only is overexpression implicated in cancer, but extreme overexpression typically corresponds to poor clinical prognoses in ovarian and colorectal carcinoma, and head and neck, lung and pancreatic cancers [46]. In 2003, Savellano and Hasan solved an important issue in PIC synthesis that had been preventing its widespread exploration for medical applications, namely aggregation of the PS leading to aggregation of the PIC [28]. Three mAb lysines were PEGylated using a 10 kDa branched PEG, which enhanced PIC adduct water solubility. This underscores a chief complication of PIC syntheses: whereas the best PS are hydrophobic and lipophilic, antibodies must remain hydrophilic. Nevertheless, Savellano and Hasan circumvented these solubility issues through the attachment of benzoporphyrin derivative (verteporfin) (Figure 2) to C225 anti-EGFR chimeric mAb, in 75% yield for a molar ratio of 2 PS/ mAb, and approximately 45% yield for a molar ratio of 10 PS/mAb. Due to the efficacy of this PEGylation technique other groups have since exploited it. One such example is the conjugation of 2C5 mAbs (2C5 recognizes tumor-specific surface-bound nucleosomes) to PEGylated micelles encasing meso-tetraphenylporphine [47].

PICs killed EGFR-overexpressing A-431 cells with illumination and did not effect EGFR-negative cells. While the group speculated that the PIC killing efficiency was a consequence of “modified subcellular localization characteristics,” elucidation of the site of localization was not part of the study. Taking these intriguing results into account, Savellano and Hasan continued to explore benzoporphyrin derivative C225 PICs [41]. Interestingly, on a per-mole basis, PICs were less phototoxic than benzoporphyrin derivative. They observed enhanced phototoxicity with increased incubation times and calculated that subcellular localization is key for efficient irradiation of EGFR-expressing cells.

The aforementioned findings inspired subcellular localization studies. In 2012 the Hasan group reported their linking of a benzoporphyrin derivative monoacid A NHS ester to a

PEGylated cetuximab mAb [48]. Altered subcellular localization and phototoxicity of EGFR-positive cells and no effect of the PIC on EGFR-negative cells was observed. Conjugation did not alter mAb-binding specificity. Remarkably, whereas the benzoporphyrin derivative monoacid A localized in the mitochondria, the PIC benzoporphyrin derivative monoacid A localized in the lysosomes in an OVCAR-5 ovarian cancer cell line, which corresponds to the subcellular localization site for free cetuximab. The PIC of this study also inhibited phosphorylation of EGFR, as well as downstream molecules involved in angiogenesis. That is, the Hasan-group PIC not only leads to destruction of the lysosome (leading to leakage of lytic enzymes) but also destroys the function of EGFR.

Vrounraets *et al.* coupled aluminum (III) phthalocyanine tetrasulfonate and meta-tetrahydroxyphenylchlorin PS to various mAbs, including those recognizing the EGFR [49]. Both types of conjugates were assayed for efficiency in the photodestruction of five different squamous cell carcinoma cell lines. While the phthalocyanine immunoconjugates were effective in the photo-destruction of all cell lines, the chlorin immunoconjugates were not. In terms of distribution, PS attachment to mAbs did not alter binding efficiency of the mAb. Even though immunospecificity of PICs and free mAbs did not differ, the binding characteristics of PICs and mAbs did differ. PIC efficacy was correlated with binding capacity (surface adhesion as well as internalization capacity) and was less correlated with internalization capacity, indicating that superficial destruction of the cell membrane was responsible for PIC-mediated death upon irradiation.

Finally, Hemming *et al.* developed a tumor-specific mAb benzoporphyrin derivative targeted against the EGFR [27]. The group used a Syrian golden hamster cheek pouch model of squamous cell carcinoma (via implantation of anthracene-impregnated silicone) to test the efficacy of PICs compared with free benzoporphyrin derivative. Interestingly, hamsters treated with the tumor-specific anti-EGFR PIC with illumination one-twentieth that of benzoporphyrin derivative only showed an 80% 1-month cancer-free survival that was not statistically significant compared with mice exposed to benzoporphyrin derivative alone.

PICs against HER2

Kuimova *et al.* targeted HER2 using PICs consisting of preclinical pyropheophorbide-a or verteporfin, conjugated to single chain fragments of mAbs (Figure 2) [50]. High selectivity was noted for HER2-positive cells and no accumulation was noted for HER2-negative cells. PICs drastically increased the rate of cellular uptake by sixfold and there was greater retention of the verteporfin PIC compared with free verteporfin. Exchange of growth medium caused free PS leakage; however, this exchange led to no change in retention of PICs. While the authors never characterized the site of localization, they speculate that cell uptake was mediated by receptor-mediated endocytosis.

An extremely elegant four-component antibody-phthalocyanine-PEG-gold nanoparticle conjugate targeting HER2 was described by Stuchinskaya *et al.* in 2011 [51]. The use of gold was justified by its negligible toxicity and the facile means by which self-assembly of thiolates on the gold surface is achieved. Using the indirect approach of PS linkage to mAbs, gold nanoparticles (4nm) were stabilized with a self-assembled layer of a zinc-chelating phthalocyanine PS and a heterobifunctional PEG. A terminal carboxy moiety on the PEG bound to the gold nanoparticles was then covalently linked to the anti-HER2 mAbs. The nanoparticles selectively targeted breast cancer cells overexpressing HER2 via photodynamic inactivation. Nanoparticles localized inside of SK-BR-3 cells (evaluated via confocal microscopy and fluorescence of the PS). While intracellular uptake was noted, the specificity of localization was not elucidated.

Savellano *et al.* constructed PICS by conjugating pyropheophorbide-a with anti-HER2 mAbs HER50 and HER66 [52]. HER66 was a superior mAb for PIC conjugation, as evaluated by PIC analysis and *in vitro* investigations. On average, ten pyropheophorbide molecules conjugated to a single HER66 mAb molecule, while only 7.5 pyropheophorbide molecules conjugated to a single HER50 mAb molecule. In competition studies between PICs and free mAbs, the HER66 PIC localization was only slightly altered in the presence of free HER66. On the other hand, HER50 PICs binding efficacy was more severely altered by the presence of free HER50. This strongly suggests that HER50 PIC synthesis may alter important structural motifs on the HER50 mAb, thus altering antigen-binding specificity. This is less likely the case in the preparation of HER60 PICs.

Carcinoembryonic antigen

The CEA is a glycoprotein involved in cell adhesion. CEA is necessary for fetal development and its production stops well before birth. CEA expression is present in colon carcinoma cells and is used for the diagnosis of colorectal, gastric, pancreatic, lung, breast and medullary thyroid carcinoma, and various adenocarcinomas [53]. Because CEA is not expressed in healthy tissue, development of PICs targeting CEA has attracted much attention. Moreover, CEA is an interesting target in that, unlike other antigen targets for PICs, CEA is considered to be a non-internalizing antigen. That is, photodynamic inactivation results in the extracellular formation of ROS, and destruction of lipid membranes via lipid oxidation and peroxidation, in turn leading to cellular leakage.

Hudson and colleagues performed an elaborate study conjugating two water-soluble PS, 5-(4-isothiocyanatophenyl)-10,15,20-tri(3,5-dihydroxyphenyl)porphyrin and 5-(4-isothiocyanatophenyl)-10,15,20-tris-(4-*N*-methylpyridiniumyl)porphyrintrichloride (Figure 2) to various mAbs using the isothiocyanate linking approach, which forms stable thiourea bonds between PS and mAb [37]. The mAbs in the study were the murine mAb 35A7 (anti-CEA), FSP 77 (anti-EGF HER2, which is overexpressed in ovarian and breast cancers, and anti-epithelial cell-adhesion molecule, which is expressed in almost all carcinomas) and 17.1A (anti-EpCAM, where EpCAM is an epitope frequently expressed in colorectal cancers). Retention of antigen binding was demonstrated using flow cytometry. Conjugation of PS to the mAb against an epithelia cell-adhesion molecule led to a significant decrease in the amount of PS required for inhibition of Colo 320 cell growth. In addition, the mAb against CEA was more effective than free PS. That being said, PICs engineered to recognize internalizing targets (HER2 and epithelial cell-adhesion molecule) were more effective than PICs engineered to recognize CEA.

Hudson also performed an extensive bio-distribution study using ¹³¹I-labeled mAbs in PICs. PICs had biodistribution values comparable with the unconjugated mAbs, representing a high retention of mAb PK. A high uptake of PICs in the blood was observed when targeted against HER2. When PS loading on mAbs was increased using the latter PS (Figure 2) (with methylpyridiniumyl-moieties), uptake in target tumor tissue, as well as other organs, decreased significantly. This is because the PS used in this study possess cationic charges and an increase in mAb cationic charge reduces serum half-life to 5% of the unmodified mAb [54].

A team led by Carcenac and Pélegrin investigated a phthalocyanine-anti-CEA mAb PIC [55]. Using a five-carbon spacer chain, aluminum tetrasulfophthalocyanine (Figure 2) was covalently coupled to the 35A7 mAb (an anti-CEA mAb) in molar ratios of PS/mAb of 5–16/1. Coupling did not modify 35A7 binding efficiency. PICs were evaluated *in vivo* using a nude mouse carcinoma xenograft model. Testing PICs with 5, 12 and 16 mol PS/mAb, it was determined that the tumor uptakes of the injected dose, per gram of tumor tissue, were 35, 40 and 32%, respectively. PICs with 5, 12 and 16 mol PS/mAb were bound to tumor

tissue 96, 104 and 91% compared with controls. Biodistribution studies were carried out and it was determined that there was almost no change in biodistribution patterns between PICs and free mAb; tumor/normal tissue uptake ratios were 1.8, 7 and 30 for blood, muscle and liver, respectively.

Sonodynamic therapy (SDT) was born out of PDT and uses a sonosensitizer (frequently similar in structure to tetrapyrrole-based PS) and ultrasound, rather than a regular PS and light, to generate ROS [56]. SDT is obviously more advantageous than PDT in terms of tissue targeting potential, as ultrasound penetrates tissue much more deeply than does visible light. Moreover, unlike PDT, whose embrace by clinicians, patients and approval boards is hindered by reluctance to accept laser technologies, SDT relies on ultrasound technologies that are already routinely used in the clinical setting. Much like the conjugation of PS to mAbs to make PICs, sonosensitizers have been linked to anti-CEA mAbs for the creation of sonoimmunoconjugates (SICs). Abe *et al.* conjugated a gallium porphyrin with anti-CEA antibodies [57]. Unsurprisingly, SIC binding affinity to CEA was no different than the binding affinity of free mAbs. Ultrasound irradiation combined with SICs was more effective in the destruction of cancerous cells compared with free gallium porphyrin alone. An *in vivo* murine xenograft model of human gastric adenocarcinoma led to a marked reduction in tumor cells compared with free gallium porphyrin and ultrasound.

PICs against other cancer-related antigens

Donald *et al.* generated PICs from ambiguous mAbs raised against MCF-7 breast cancer cells, yet the resultant mAbs demonstrated “a specificity for an antigen common to many epithelial cells lines” and they noted that the mAb has a particular avidity for squamous cell carcinoma [58]. Conjugating the impure mixture of hematoporphyrin derivative and irradiating at 630 nm, the authors noted a reduction in cancerous cell viability by 5 log₁₀. PICs localized on the microvilli. Using an *in vivo* model, squamous cell carcinoma was grown subcutaneously in a population of nude mice, and mice were injected with the aforementioned PICs. At 24 h, PIC retention was 6% in the skin (compared with 15% retention in malignant tissue), but after 48 h these dropped to 2.5 and 13%, respectively.

PICs for the treatment of squamous cell carcinomas were also developed by Jiang and Levy [26,59,60]. Other PICs have targeted various antigens characteristic of cancerous cells, as well as infectious agents (i.e., bacteria). Herein we describe some of the more interesting and diverse developed PICs.

VEGF is an angiogenic protein responsible for malignant cell-induced angiogenesis. VEGF overexpression is implicated in poor breast cancer prognosis and is linked in metastasis; thus, VEGF is an ideal anticancer target and in 2004 the first anti-VEGF antibody bevacizumab was approved by the FDA [61]. Hematoporphyrin was successfully conjugated to anti-VEGF antibodies and tested against mouse Lewis carcinoma [62]. The authors irradiated mice 24-h post-incubation with the hematoporphyrin derivative. Seven weeks post-therapy, when all mice of the control group and free PS group had died, 40% of mice subjected to PIC survived and 26% of mice exposed to 5-aminolevulinic acid PDT survived. That is, the PIC proved itself superior to both types of PDT.

CA-125, also known as mucin 16, has been targeted for the development of PICs suitable for ovarian cancers. The Hasan group used a Balb/c athymic (immunocompromised) mouse model of ovarian cancer to evaluate the anti-CA-125 mAb (known as OC125) conjugated to PS to form PICs [63]. A chlorin PS conjugated to OC125 was shown to be effective in the photoirradiation of malignant cells both *in vitro* and *in vivo*. Comprehensive biodistribution analyses were performed: peak tumor concentrations of PICs were reached 24 h post-intraperitoneal injection. Tumor/non-tumor ratios were 6.8 for blood, 6.5 for liver, 7.2 for

kidney, 5.7 for skin and 3.5 for the intestine. The Hasan group followed this work with a second study, demonstrating that several irradiation sessions with PICs enhanced therapeutic outcome [64]. For example, mice incubated with PICs via intraperitoneal injection with three irradiation (656 nm) sessions had a 40% survival post-50 days of injection, and mice exposed to four irradiations sessions had a 58% survival rate [64].

PDT has not been seriously considered for the treatment of hepatic cancers, as typically PS uptake in hepatic tumor tissue is comparable with that of surrounding liver tissue. In an effort to circumvent this issue, Hamblin and Hasan developed a PIC using murine mAb 17.1A (targeted against HT-29 cells) and chlorin e6 [38]. Intravenous injection of anionic and cationic variants of the PIC had profound differences: the anionic PIC variant was fivefold more effective at tumor localization post 3 h of injection with a hepatic tumor/normal hepatic tissue uptake ratio of 2.5. Cationic species had a high uptake in the lungs, emphasizing the disadvantage of cationic PIC underscored elsewhere in this review. The PIC tumor/skin accumulation ratio was very high(>38).

PICs against microbial antigens

This review has so far focused on the use of PICs for the treatment of cancerous conditions. This may reflect the fact that PDT was initially explored in the treatment of malignancies. Antimicrobial PDT, still in its infancy, will undoubtedly play a role in the 21st century for overcoming antimicrobial resistance and killing antimicrobial-resistant pathogens. PICs have been developed for antimicrobial PDT, although examples of these technologies are few and far between. Lu *et al.* developed a PIC by conjugation of a Sn-chlorin e6 PS to anti-*Pseudomonas aeruginosa* mAb [65]. PS/mAb molar ratios from 1.6 to 10 were achieved, and these PICs only possessed slightly reduced quantum yields of $^1\text{O}_2$. All conjugates possessed significant cytotoxicity against *P. aeruginosa* upon irradiation, and biodistribution studies indicated that PICs with a triethanolamine ligand (note that this is cationic) possessed significant serum retention 24 h post-injection, but conjugates lacking this ligand cleared more quickly. *In vivo* specificity was confirmed when rats that had been infected with Fischer immunotype I *P. aeruginosa* were injected with PICs. The authors noted that the *in vivo* behavior of the PIC was remarkably similar to that of free mAb, which is consistent with almost all findings listed in this review. The *bis*(triethanolamine) ligand stabilized the Sn-chlorin e6 against enzymatic degradation and dimerization and 120 h post-injection the PICs were still biologically active and structurally intact. Further studies of Sn-chlorin e6 PICs have been carried out elsewhere [66].

PICs in the clinic: past investigations & future endeavors

Although PIC investigations are certainly still in their infancy and the impact of PIC technologies in the treatment of malignant disease has yet to be realized, interest in this field is rapidly growing. That being said, without clinical and pre-clinical investigations, the field has no chance of gaining widespread acceptance by the medical community, let alone capturing the attention of medical professionals.

Due to the infancy of PIC investigations there have only been scarce evaluations of PICs in clinical and preclinical investigations. To date, only one clinical PIC investigation has been carried out by Schmidt *et al.* in Germany in the early 1990s [67]. Unfortunately, Schmidt's papers have been published in German, thus impeding their widespread acceptance and circulation in the PIC community. First, Schmidt and colleagues conjugated phthalocyanine with a mAb against ovarian cancer cells and demonstrated the selectivity of the conjugate in *in vitro* investigations [68,69]. Following this work, three women with advanced ovarian cancer (stage III, according to The Federation of Obstetricians and Gynecologists classification) were treated with the conjugates [67]. There was evidence of some response

after administration of PICs and illumination of tumor cells. Ultramicroscopic analysis demonstrated a selective devitalization of tumor cells compared with surrounding tissue. Although these results are promising, PICs are still far from clinical applications.

Schmidt *et al.*'s studies beg the question of what properties an ideal, clinical PIC should possess. Although clinical PS properties have been stipulated extensively, thus inspiring and guiding efforts to synthesize novel PS, this is less the case for the PIC community. First and foremost, an ideal PIC would possess all of the properties of an ideal PS, and conjugation of PS with mAbs should not affect PS absorption spectra nor mAb binding affinity. Nevertheless, this has been particularly challenging. Clinical mAbs, too, must meet strict criteria before clinical administration and, to date, only 16 mAb therapeutics have reached blockbuster status in the medical world. The properties of an ideal mAb include high antigen-binding specificity (which results in negligible side effects), a short pharmacological half-life ranging from 10 min to 24 h, and easy solvation in saline solutions for intravenous injection. Unfortunately, conjugation of PS to mAbs does slightly alter PS spectral properties and mAb binding affinity, as demonstrated throughout this review. The use of linkers has attempted to escape spectral alteration of PS upon modification, and mAb binding is sometimes negligible. In the case of mAb modification, a loss of binding specificity may be inescapable: modification of the chemical properties of mAbs may irreversibly alter the folding nature of the mAbs. PIC generation is complicated in that a perfect PS (one that meets all of the requirements of the ideal PS) has yet to be created and the PIC community has yet to explicitly define the properties of ideal PICs.

Future perspective

Advances are constantly being made in PS immunoconjugate synthesis, purification, characterization and bioanalysis. Undoubtedly these advances have improved the purity, quality and structural certainty associated with recently reported PIC; these qualities may not have been of equivalent standard in papers reported in the past years. Appropriate means of independently analyzing the various components of the PIC (antibody, PS and linker) are needed to accurately determine the PK, PD and biodistribution of PICs, which will of course be critical if these constructs are ever to be tested clinically. The rise of clinical mAb therapy (adalimumab, alemtuzumab, bevacizumab, cetuximab, natalizumab and ranibizumab, to name only a few) suggests that the prospect of PICs emerging into clinical trials may not be as distant as was once thought, and that bioanalysis considerations may be the key advance required for this to happen.

Key Terms

Photodynamic therapy	Combination of a nontoxic dye with harmless visible light that produces reactive oxygen species and kills cells
Photosensitizers	Highly colored molecules often chemically related to porphyrins, which are able to localize in tumors and mediate photodynamic therapy
Photoimmunoconjugate	Covalent conjugate between a monoclonal antibody and a photosensitizer to increase the targeting ability of photodynamic therapy
Reactive oxygen species	Singlet oxygen or hydroxyl radical produced during photodynamic therapy that can oxidize proteins and lipids

Photoimmunoconjugate bioanalysis	Analytical chemistry techniques employed to characterize photoimmunoconjugates such as PAGE, HPLC and MALDI
Tumor-associated antigen	Cell surface molecule, such as a growth factor receptor, that is overexpressed on cancer cells and is recognized by an antibody

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Executive summary

- Photodynamic therapy involves the combination of nontoxic photosensitizers, visible light of the appropriate wavelength to be absorbed, and molecular oxygen.
- The long-lived excited triplet photosensitizer produces reactive oxygen species that destroy cells.
- Although photodynamic therapy has intrinsic dual selectivity, researchers are constantly searching for ways to improve tumor-targeting ability.
- Conjugating photosensitizers to monoclonal antibodies that recognize tumor-associated antigens has become a widely studied approach.
- Either direct conjugation via monoclonal antibody amino groups or indirect conjugation via an intermediate linker have been reported.
- The molecular targets that have attracted attention include: EGFR, HER2, carcinoembryonic antigen, VEGF and bacterial antigens.
- The bioanalytical methods employed to characterize photoimmunoconjugates include PAGE, HPLC and MALDI-MS.

Figure 1. Modified Jablonski diagram

A PS, here represented as the cationic phenothiazinium dye methylene blue, is excited to the excited singlet state where it relaxes to the excited triplet state. Thermodynamically favored reactions with ground (triplet state) oxygen ensue, resulting in the formation of radicals and $^1\text{O}_2$. The superscript numbers in the figure refer to spin multiplicity of highest occupied molecular orbital; 1 = singlet; 3 = triplet.

*Signifies excited state (as compared with ground state).

PS: Photosensitizer.

Figure 2. Major porphyrins used for preparing photoimmunoconjugates and conjugate linkages Hematoporphyrin, benzoporphyrin derivative and pyropheophorbide-a are examples of tetrapyrrole photosensitizers. Different direct chemical linkages and indirect linker-based conjugation chemistries are listed.

Figure 3. Major target groups for photoimmunoconjugates

PICs bind to antigens specifically expressed on the surface of cancerous/undesired cells. Irradiation with light before or after internalization leads to membrane or organelle damage, respectively. Additional ovals in bottom cells indicate that when the PIC is internalized the damage is intracellular.

PIC: Photoimmunoconjugate.